Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 24-35 are pending in the application, with 24, 25 and 26 being the independent claims. Claims 24-26 are sought to be amended. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Claims 24-26 have been amended to supply a point of reference and to make explicit what is implicit.

The specification has been amended to delete what is believed to be an inadvertent error. This is further described at page 9 of the reply. The amendment to the specification is believed to introduce no new matter, and entry of the amendment is respectfully requested

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 24 to 35 under 35 U.S.C. § 112, first paragraph as allegedly containing "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." (Paper No. 20040826, p.2). Applicants respectfully traverse the rejection.

The Examiner states that the claim limitation "'said second cell line also has inhibited Gs signaling of u-PA activity' has no basis in the instant application as filed. This language was not present in the application as filed and constitutes new inventive concept because it does not inherently flow from the original disclosure." (Paper No. 20040826, p. 2). The Examiner further states that the specification described

> "AB45 cells" in which human PTHRs (370,000/cell) were co-expressed with REV AB, a dominant-negative inhibitor of both basal and hormone-stimulated protein kinase A (PKA). The text on page 10 of the specification states that "in LLC-PK1 cells, both the PKA and PKC pathways are linked to U-PA production" and that "Gs and Gq proteins activate the PKC pathway and thereby increase U-PA production in LLC-PK1 cells." Therefore, one would not conclude that AB45 cells had "inhibited Gs signaling of u-PA activity" since Gs activates the PKC pathway and it was the PKA pathway that was inhibited by the heterologous REV AB gene in those cells.

(Paper No. 20040826, p. 3). Applicants disagree.

The sentence on page 10 to which the Examiner refers contains an inadvertent error. The specification describes receptors which couple to both Gs and Gq proteins. It was known in the art at the time of the earliest priority date, July 31, 1997, that Gs coupled receptors activate the PKA pathway and that Gq coupled receptors activate the PKC pathway. Applicants submit as Exhibit A, several illustrative figures from Bruce Alberts *et al.*, Molecular Biology of the Cell, (3d ed. 1994), which explains the model of the Gs and Gq coupled pathways. The Gs protein is coupled to the activation of adenyl cyclase which produces cyclic AMP. (*See* Exhibit A, figure 15-23). Cyclic AMP then activates the cyclic AMP dependent protein kinase (PKA). (*See* Exhibit A, figure 15-24). The Gs protein is not coupled to the PKC pathway. Exhibit A also illustrates that the Gq protein is coupled to the activation of phospholipase C-β which results in the

cleavage of phosphatidylinositol biphosphate (PIP_2) and the generation of inositol triphosphate (IP_3) and diacylglycerol. (*See* Exhibit A, figure 15-33). The diacylglycerol, in combination with Ca^{2+} and phosphatidlyserine activate protein kinase C (PKC) (*See id.*) Thus, Applicants assert that one skilled in the art reading the specification would have recognized the sentence "Gs and Gq proteins activate the PKC pathway and thereby increase production in LLC-PK1 cells" as an error. One of skill in the art reading the disclosure would know that Gs is coupled to the pathway which activates PKA, while Gq is coupled to the pathway that activates PKC, as shown in Exhibit A. Furthermore, as discussed in more detail below, one of skill in the art reading the entire specification would conclude that the AB45 cell line has inhibited Gs signaling of u-PA activity. Thus, the language referred to by the Examiner in the rejection represents an inherent characteristic of the cell line.

The Examiner has also stated that

> [i]f Applicant can identify that portion of the instant specification, as filed, which serves as a basis for the limitation "said second cell line also has inhibited Gs signaling of u-PA activity" they are encouraged to do so in response to this action. Those portions of the specification that were identified by Applicant in the correspondence of 21 June 2004 as providing support for the new claims have been carefully reviewed and they do not support the limitation "said second cell line also has inhibited Gs signally of u-PA activity."

(Paper No. 20040826, p.3). Applicants disagree and wish to bring to the Examiner's attention Figure 7, which was cited as support in the Reply and Amendment filed June 21, 2004. Figure 7 shows the results of an experiment in which secretion of u-PA was measured after the addition of various agonists to AB45 cells (LLC-PK1 cells which express a dominant negative inhibitor of PKA). The results in Figure 7 illustrate that

when the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is used as the agonist of human PTHR, the AB45 cells secrete almost three times the amount of u-PA as measured by u-PA activity. TPA is a PKC specific agonist and thus only activates the Gq coupled PKC pathway. (*See* page 26, lines 14-15 and page 27, lines 1-3). However, when 8-bromoadenosine 3', 5'-monophosphate (8BrcAMP), a PKA specific agonist, is used in the same experiment, u-PA production is almost identical to the amount of u-PA produced by the control cells in which no agonist was used. (*See* page 26, line 29 - page 27, line 3.) These data indicate that PKA signaling is blocked in the AB45 cell line. Since it is believed that Gs does not signal through the PKC pathway and the results described above indicate that the ability of the AB45 cells to produce u-PA through the Gs linked PKA pathway has been inhibited, one of skill in the art would conclude, in combination with the knowledge in the art discussed above, that the AB45 cells have inhibited Gs signaling. Therefore, Applicants assert that the specification does indeed provide written description to support the limitation "said second cell line also has inhibited Gs signaling of u-PA activity" and that one skilled in the art reading the sentence on page 10 would have concluded that it was in error.

Solely, to expedite prosecution and not in any way acquiescing to the Examiner's rejection, Applicants have amended page 10 of the specification to delete the sentence "Gs and Gq proteins activate the PKC pathway and thereby increase U-PA production in LLC-PK1 cells." Applicants believe that the deletion of this sentence does not constitute new matter, rather the amendment clarifies what is believed to be an error in the specification based on information that was well known in the art at the time the earliest priority application was filed and the information contained in the specification when

considered in its entirety. Thus, Applicants consider the rejection moot and respectfully request that the rejection is withdrawn.

Rejections under 35 U.S.C. § 112, 2nd paragraph

The Examiner has rejected claims 24 to 35 under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. Applicants respectfully traverse the rejection.

Specifically, the Examiner states that "the limitation 'has inhibited Gs signaling of u-PA activity' requires a point of reference, such as 'relative to said first cell line', and none is given." (Paper No. 20040826, p. 4). Applicants respectfully disagree.

Solely in an effort to expedite prosecution and without acquiescence in the propriety of the rejection, Applicants have amended claims 24-26, from which 27-35 depend, to state "relative to said first cell line" as suggested by the Examiner.

Thus, Applicants consider this rejection moot and respectfully request that the rejection is withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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MOLECULAR BIOLOGY OF THE CELL

THIRD EDITION

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

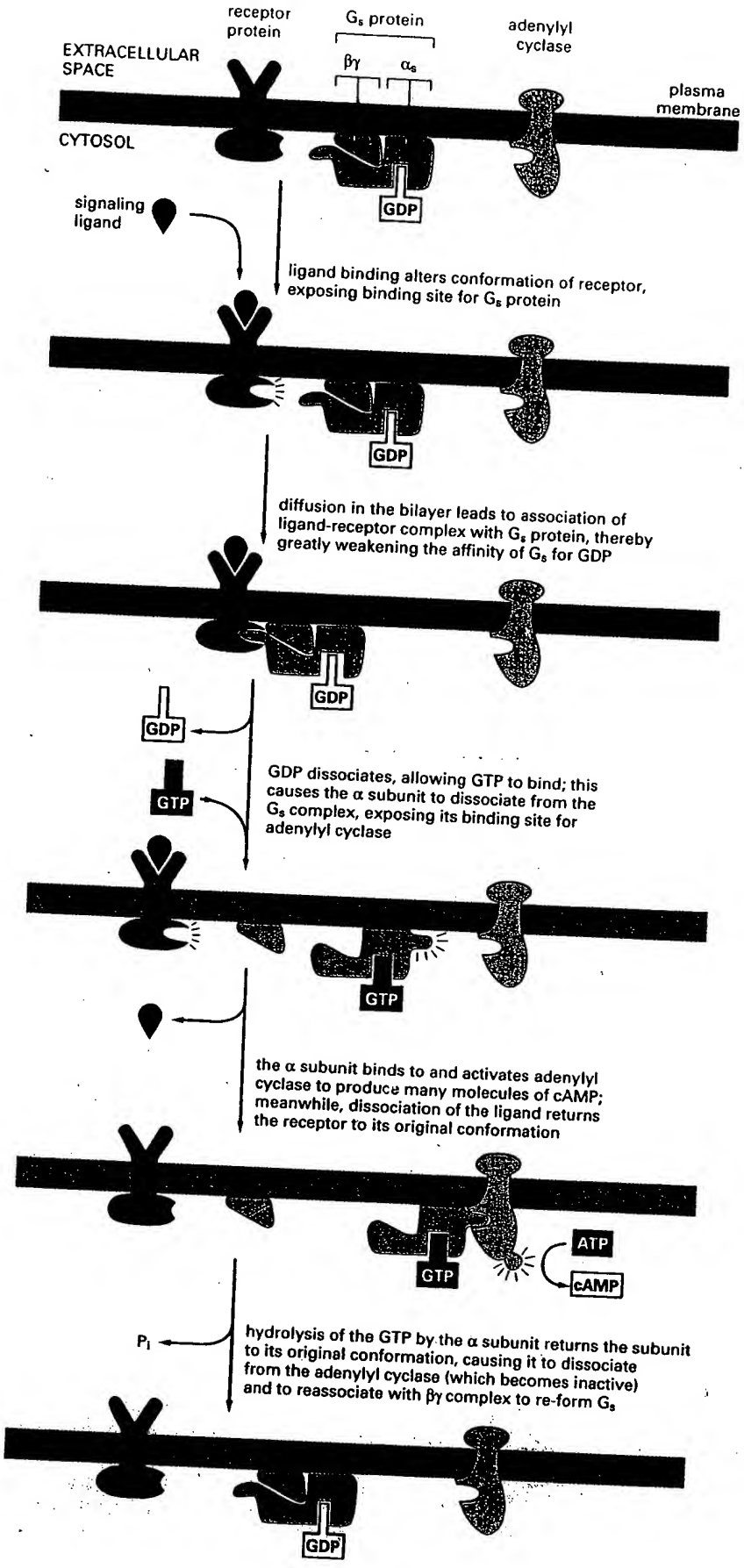

Figure 15–23 A current model of how G_s couples receptor activation to adenylyl cyclase activation. As long as the extracellular signaling ligand remains bound, the receptor protein can continue to activate molecules of G_s protein, thereby amplifying the response. More important, an α_s can remain active and continue to stimulate a cyclase molecule for many seconds after the signaling ligand dissociates from the receptor, providing even greater amplification.

Whereas cholera toxin catalyzes the ADP ribosylation of α_s and thereby inactivates the GTPase activity of α_s, **pertussis toxin,** made by the bacterium that causes pertussis (whooping cough), catalyzes the ADP ribosylation of α_i. The ADP ribosylation of α_i prevents the G_i complex from interacting with receptors, and so the complex remains bound to GDP and is unable to inhibit adenylyl cyclase or open K^+ channels.

The trimeric G proteins are remarkably versatile intracellular signaling molecules. In the examples considered so far, either the α subunit or both the α and the βγ subunits are the active components. But in other cases receptors are coupled to their target proteins only by the released βγ complex. Moreover, βγ complexes can also act as conditional regulators of effector proteins: they can enhance the activation of some forms of adenylyl cyclase, for example, but only if the cyclase has already been activated by α_s.

Cyclic-AMP-dependent Protein Kinase (A-Kinase) Mediates the Effects of Cyclic AMP [16]

Cyclic AMP exerts its effects in animal cells mainly by activating the enzyme **cyclic-AMP-dependent protein kinase (A-kinase),** which catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected proteins. The amino acids phosphorylated by A-kinase are marked by the presence of two or more basic amino acids on their amino-terminal side. Covalent phosphorylation of the appropriate amino acids in turn regulates the activity of the target protein.

A-kinase is found in all animal cells and is thought to account for all of the effects of cyclic AMP in most of these cells. (The only other known function of cyclic AMP in animals is to regulate a special class of ion channels in smell-responsive olfactory neurons, as we discuss later.) The substrates for A-kinase differ in different cell types, explaining why the effects of cyclic AMP vary depending on the target cell.

In the inactive state A-kinase consists of a complex of two catalytic subunits and two regulatory subunits that bind cyclic AMP. The binding of cyclic AMP alters the conformation of the regulatory subunits, causing them to dissociate from the complex. The released catalytic subunits are thereby activated to phosphorylate specific substrate protein molecules (Figure 15–24).

Cyclic-AMP-mediated protein phosphorylation was first demonstrated in studies of glycogen metabolism in skeletal muscle cells. Glycogen is the major storage form of glucose, and both its synthesis and degradation in skeletal muscle cells are regulated by adrenaline. When an animal is frightened or otherwise stressed, for example, the adrenal gland secretes adrenaline into the blood, "alerting" various tissues in the body. Among other effects, the circulating adrenaline

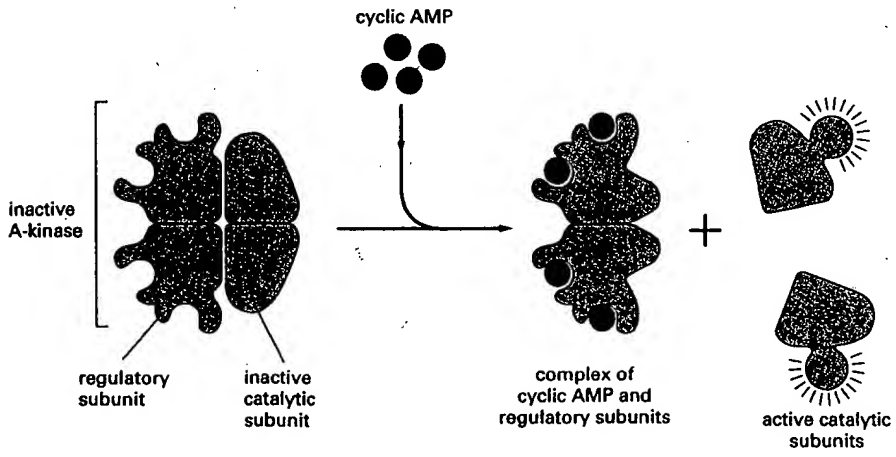

Figure 15–24 The activation of cyclic-AMP-dependent protein kinase (A-kinase). The binding of cyclic AMP to the regulatory subunits induces a conformational change, causing these subunits to dissociate from the complex, thereby activating the catalytic subunits. Each regulatory subunit has two cyclic-AMP-binding sites, and the release of the catalytic subunits requires the binding of more than two cyclic AMP molecules to the tetramer. This greatly sharpens the response of the kinase to changes in cyclic AMP concentration, as we discuss later. There are at least two types of A-kinase in most mammalian cells: type I is mainly in the cytosol, whereas type II is bound via its regulatory subunit to the plasma membrane, nuclear membrane, and microtubules. In both cases, however, once the catalytic subunits are freed and active, they can migrate into the nucleus (where they can phosphorylate gene regulatory proteins), while the regulatory subunits remain in the cytoplasm. The three-dimensional structure of the protein kinase domain of the A-kinase catalytic subunit is shown in Figure 5–12.

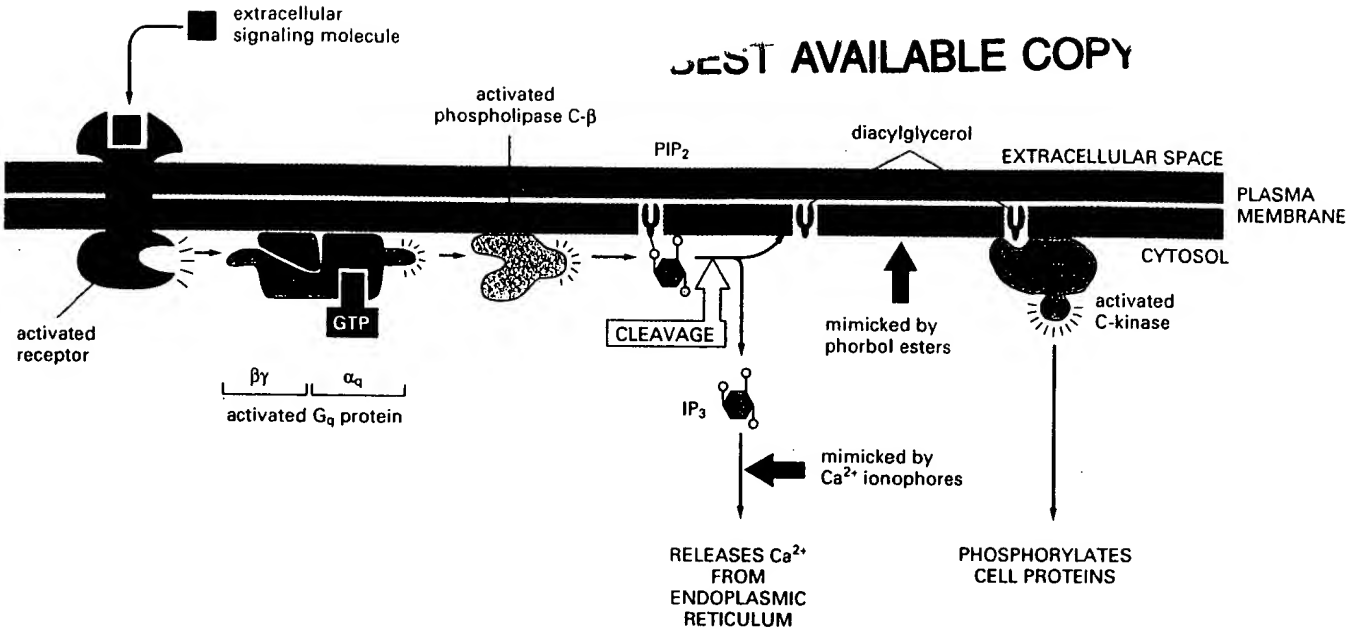

Figure 15–33 The two branches of the inositol phospholipid pathway. The activated receptor binds to a specific trimeric G protein (G_q), causing the α subunit to dissociate and activate phospholipase C-β, which cleaves PIP_2 to generate IP_3 and diacylglycerol. The diacylglycerol (together with bound Ca^{2+} and phosphatidylserine—not shown) activates C-kinase. Both phospholipase C-β and C-kinase are water-soluble enzymes that translocate from the cytosol to the inner face of the plasma membrane in the process of being activated. The effects of IP_3 can be mimicked experimentally in intact cells by treatment with Ca^{2+} ionophores, while the effects of diacylglycerol can be mimicked by treatment with phorbol esters, which bind to C-kinase and activate it.

rectly. Using these reagents, it has been shown that the two branches of the pathway often collaborate in producing a full cellular response. A number of cell types, for example, can be stimulated to proliferate in culture when treated with both a Ca^{2+} ionophore and a C-kinase activator but not when they are treated with either reagent alone.

Calmodulin Is a Ubiquitous Intracellular Ca^{2+} Receptor [24]

Since the free Ca^{2+} concentration in the cytosol is usually $\leq 10^{-7}$ M and generally does not rise above 6×10^{-6} M even when the cell is activated by an influx of Ca^{2+}, any structure in the cell that is to serve as a direct target for Ca^{2+}-dependent regulation must have an affinity constant (K_a) for Ca^{2+} of around 10^6 liters/mole. Moreover, since the concentration of free Mg^{2+} in the cytosol is relatively constant at about 10^{-3} M, these Ca^{2+}-binding sites must have a selectivity for Ca^{2+} over Mg^{2+} of at least 1000-fold. Several specific Ca^{2+}-binding proteins fulfill these criteria.

The first such protein to be discovered was *troponin C* in skeletal muscle cells; its role in muscle contraction is discussed in Chapter 16. A closely related Ca^{2+}-binding protein, known as **calmodulin,** is found in all eucaryotic cells that have been examined. A typical animal cell contains more than 10^7 molecules of calmodulin, which can constitute as much as 1% of the total protein mass of the cell. Calmodulin functions as a multipurpose intracellular Ca^{2+} receptor, mediating many Ca^{2+}-regulated processes. It is a highly conserved, single polypeptide chain of about 150 amino acids, with four high-affinity Ca^{2+}-binding sites (Figure 15–34A), and it undergoes a conformational change when it binds Ca^{2+}.

The allosteric activation of calmodulin by Ca^{2+} is analogous to the allosteric activation of A-kinase by cyclic AMP, except that Ca^{2+}/calmodulin has no enzyme activity itself but acts by binding to other proteins. In some cases calmodulin serves as a permanent regulatory subunit of an enzyme complex, but in most cases the binding of Ca^{2+} enables calmodulin to bind to various target proteins in the cell and thereby alter their activity. When Ca^{2+}/calmodulin binds to its target protein, it can undergo a further and more dramatic change in conformation (Figure 15–34B).

Among the targets regulated by Ca^{2+}/calmodulin are various enzymes and membrane transport proteins. In many cells, for example, Ca^{2+}/calmodulin binds to and activates the plasma membrane Ca^{2+}-ATPase that pumps Ca^{2+} out of the